STUDIES ON A VIRUS CAUSING MOSAIC ON AN ORNAMENTAL CALENDULA YELLOW NET VIRUS (CYNV)

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AKIL AHMED KHAN

DEPARTMENT OF BOTANY
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Qamar A. Naqvi
Sc. Ph. D., F.P.S.I., M.N.A. Sc.
ES Paris
Associate Professor

Phone : 0091 (571) 702259 (R)
0091 (571) 401016 (O)
Plant Virology Lab.,
Department of Botany
Aligarh Muslim University

Dated 23.2.2001.

Certificate

This is to certify that the dissertation entitled "Studies on a virus causing mosaic on an ornamental calendula yellow net virus (CYNV)" submitted to the Aligarh Muslim University, Aligarh in partial fulfilment of the requirements for the award of the degree of Master of Philosophy is a faithful record of the bonafide research work carried by Mr. Akil A. Khan. No part of this dissertation has been published or submitted for any other degree or diploma.

(Qamar A. Naqvi)

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Akil Ahmed Khan

Dedicated

To

My Parents

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Chapter 1
Introduction

Flowers are symbol of beauty, love and tranquility. They form the soul of garden and convey the message of nature to man. Flowers have served as an invaluable aid for personal make up, an excellent offering to God, and source of inspiration to poets. Since the begining of civilization man has been accustomed to use flowers to make his festivals more festive. In Sanskrit literature of the ancient part flowers epigrams have been used copiously to interpret romantic situation. Being so much involved with man's daily life, flowers have developed a language of their own and can speak volumes of human sentiments.

Calendula officinalis belongs to family compositae / Astraceae. The family is generally regarded as occupying the highest position among the angiosperm or at least among the dicots in the course of evolution as is evident by the presence of largest number viz. 2000 species of 950 genera and the cosmopolitan distribution from the arctic to the tropical zone. There are about 25 species of Calendula but only two species are found in India i.e. *C. officinalis* and *C. arvense*. Genus Calendula is distributed in South Europe, Northern Africa and Western Asia. It is cultivated in temperate regions around the world.

Calendula is easily propagated from seeds. It flourishes in almost all type of soils. The plant is annual or perennial herb with 1-2 feet height. The ligulate florate are bright or orange or yellow in colour.

Economically the Calendula is of great importance. The flowers contain an amorphous bitter principle calendulin, a yellow test less substance analogous to bassorin, trace of an essential oil, aleanolicacid, a gum, a sterol ($C_{26}H_{44}O_2$ m.p.209^0) cholestrol, esters of lauric, myristic palmitic, stearic and pentadecyclic acid faradiol m.p. 211° and arnidiol m.p. 257°.

The pigment is a mixture of β-carotene, lycopene, violaxanthin and other xanthophyll. The plant contain salicyclic acid (0.34 mg/kg) in fresh material, and insulin is present in the roots.

Calendula officinalis, also known as pot marigold, is a mild aromatic with diaphoretic, diuretic and stimulant properties. A mixture of dried flowers is administered in the treatment of amenrrhoea and after dilution as a lotion for sprains and bruises. Calendula is now almost absolete as a drug and is used to adulterate saffron and arnica flowers.

Like other pathogen which affect the aesthetic value of ornamental plants, viruses are of significant importance due to the absence of therapeutic control measures against them in plants. Viruses have a economic importance in floriculture due to severe losses caused by reduction in growth number and size of flowers. Thus viruses are a patent threat as well as limiting factor in floriculture.

During the survey of virus disease of ornamentals in and around Aligarh, a severe disease of virus of calendula was observed which shows yellow net symptom, reduction in size of leaves and number of flowers. Severely affected plants showed reduction in flowers yield and quality. A preliminary studies with regard to the Calendula yellow net virus (CYNV) were therefore, undertaken to establish the identity of the causal agent of this disease and characterize the virus isolate causing yellow mosaic disease in Calendula (CYNV).

Chapter 2

Review of Literature

By going through review of literature of diseases of ornamental plants, it was found that many diseases were reported on various diseases of ornamentals belonging to family Asteraceae but a very few diseases are found on *Calendula officinalis* belonging to the same family. However, the Calendula which is a common ornamental was used as a differential hosts by many authors from time to time e.g. (Smith 1936) while, studying tomato spotted wilt virus in his ornamental hosts used Calendula in host range studies. The other viruses which were screened against this host by the same author are tulip breaking, lily mosaic, rosette on yellow flat and pelagonium leaf curl.

In their report (Walker *et al,* 1941), reported that the cabbage mosaic found to be infected by two viruses, tentatively referred to as A and B. A was found to be largely responsible for the mosaic mottle while B produce vein clearing. B was largely confined to crucifers, where A causes mosaic on crucifers, tobacco delphinim, petunia, calendula and zinnia. Both A and B infect chard and spinach. Each infect cabbage independently. The disease produced by B alone is very mild, while that caused by A alone is more severe. A is rendered non infectious by exposure to a temperature of 130°F for 10 minute, while B resist temperature upto about 150°C for this period.

Attention was drawn to the increasing prevalence of carnation mosaic, streak and yellows virus in Colorado by (Runley and Thomas 1948). The total losses due to the streak and yellows complex in 1947 was amounted to more than 10% of the production. Among the plants to which this virus infected are *zinnia*, celery, sugarbeet, Calendula, pintobean (*Phaseolus vulgaris*), holy hock, white clover, sweet clover (Melilotus) and Gladioleus, white aster and *Nicotiana rustica* are liable to infection by streak. The former virus is readily dissiminated by rubbing on with the cutting knife, while both mosaic and streak are transmissible by grafting.

(Vela 1972) isolated a virus from *Calendula officinalis* produced characteristic mosaic symptoms on tobacco and *Nicotiana glutinosa*. Elongtated particles 15x730-750 mμ and amorphous inclusion bodies were observed. The distribution pattern of these was similar to those induced by the potato Y gp., but tubules similar to the once found in X-bodies from tobacco mosaic virus were also seen.

In her paper (Padma *et al.*, 1975) reported certain observation on the occurrance of mosaic disease on *Calendula officinalis*. In her report she said that the disease is recorded for the first time from India, and was sap transmissible on to *Nicotiana glutinosa* and *Chenopodium amaranticolor*. The

disease was also transmitted by grafting from calendula to calendula and also by *Aphis gossypii* and *Myzus persicae*.

(Procter 1976) in his work studies on tomato aspermy virus from *chrysanthemum indicum* L. reported TMV for the first time from Chrysanthemum in NZ for the first time in commercial garden in Christchurch, Wanganai and Auckland. The virus was transferred experimentally to *Calendula officinalis, Tropacolum-majus, aster, capsicum, chrysanthemum, Cyphomandra batacae* lettuce, tomato, *Nicotiana glutinosa, Chenopodium amaranticolor*, tobacco, *Solanum nigrum,* New Zealand spinach (*Tetragona expansa)* and Zinnia. The longevity of virus was 7-11 days and the thermal inactivation point 65°-70°C. The dilution end point was between 1-50000 and 1-100,000. the virus was transmitted by *Myzus persicae*.

In their studies of physiological studies with *Calendula officinalis* leaves infected with potato yellow dwarf virus (Dhillon et al., 1979) found that respiration of leaves infected with the new Jersey str. of PYDV was mediated through Embden-Meyerhof Parnas (EMP) and Tricarboxylic (TCA) pathways. Respiration and EMP + TCA pathway, increased in the initial stages but decreased in the later stages of disease development. Operation of the hexose monophasphate pathway could not be detected. In their studies they found chlorophyll,

total sugar, non reducing sugars, starch and total carbohydrate decreased while reducing sugar and RNA increased in infected leaves.

Two different viruses were reported from *Calendula officinalis* in Liguria by (Lisa & Della in the year 1980). It was found that the infection is caused by cucumber mosaic and turnip mosaic viruses singly or together. The infected plants showed reduced growth, various degrees of leaf mosaic on mottling and fewer marketable flowers, but no visible flower symptoms.

(Naqvi & Mahmood 1980) reported Calendula yellow net, a new cucumo virus infecting *Calendula Officinalis*. In their studies they found that the properties of this causal virus is same as the viruses belonging to this group of plant viruses.

The calendula yellow net virus was successfully purified by (Naqvi & Samad1987) by extraction with 0.05M PO_4 buffer (pH 7.5) and chloroform followed by 3 cycles of differential centrifugation.

Virus disease showing yellow net symptoms on *Calendula officinalis* L. was observed in Kasimpur, Aligarh by (Naqvi et al., 1989). The virus was found to be mechanically transmitted from infected to healthy young seedlings of *C. officinalis*. In

extract of *C. officinalis* leaves the virus lost its infectivity after 10 min. of exposure at 65°C but not at 60°C and after dilution to 10^{-3} but not to 10^{-2}. The virus was able to retain its infectivity upto 76h. when stored at room temperature (25±5°C). The virus was transmitted by two species of aphid, viz. *Aphis gossypii* Glov. and *Myzus persicae* Sulz. in persistent manner. The causal virus of Calendula yellow net disease was suspected to belong to cucumo virus group.

Chapter 3

Material & Methods

1. Maintenance of virus inoculam:

i) Raising of test plants:

All the plants were grown in clay pots 4" and 6" diameters, filled with a mixture of soil, sand and compost in a ratio of 2:1:1. The soil mixture was sterilized by autoclaving for one hour at a pressure of 20lbs per square inch. The clay pots were sterilized by rinsing in 4% formalin solution and the soil mixture autoclaved 24 hours earlier and sieved before use.

Seeds were sown in 12" clay pots for raising seedlings except for plants belonging to cucurbitaceae and leguminosae which were raised singly by direct sowing in clay pots. Seedlings were transplanted singly in clay pots of 4" and 6" diameter at 2-3 leaf stage, when they were about 2 weeks old.

For inoculations the plants were used two weeks after transplantation. All the plants were kept in an insect proof glass house.

ii) Virus culture:

Young leaves of naturally infected plant of Calendula *(C. officinalis)* showing symptoms of severe yellow net were macerated using mortar and pestle (with simultaneous addition of 0.01M phosphate buffer (pH 7.0). The macerate was filtered through double layered cheese cloth. Attempts of single lesion

inoculations were made to maintain a pure virus culture on suitable propagation host 4-5 leaf stage. Inoculations were made by weekly mechanical inoculation onto healthy plants of propagation host to increase culture. Periodic checks were made on assay host, *Chenopodium amaranticolor* to ensure biological purity of the virus.

iii) **Source of inoculum:**

Young leaves of propagation host was used as a source of inoculum, prepared by macerating them in a mortar with pestle in 0.01M phosphate buffer (pH 7.0). For each gram of infected leaves 2ml of buffer was used and the macerate was filtered through double layered cheese cloth. The sap thus obtained was used as standard inoculum (SI).

2. Transmission:

2.1 **Mechanical:** The fully expanded leaves of the plants to be inoculated will be dusted uniformly with carborundum 500 mesh as an abrasive and the standard inoculum will be applied gently but firmly on the upper surface of leaves with the help of fore-finger by keeping the other hand beneath the leaf to be inoculated. The inoculated leaves will be rinsed with gentle stream of water before the inoculum on the surface of leaves dried up. If the rate

of transmission is not promising, some chemical will be mixed with the inoculum so as to enhance the rate of transmission. Additive in the inoculum will include sodium sulfite, 2-mercapto-ethanol, ethylene diamine-tetra-acetic acid, sodium diethyl-dithiocarbomate and thio-glycollic acid either alone or in various possible combinations if needed.

2.2 **Biological:** Attempts will be made to find out the vector/s of viruses in the field, transmission by insects, soil, dodder (*Cuscuta* spp.), seeds, graft, nematode/s and pollen will be studied.

2.2.1: Insect transmission:

2.2.1 (a) Transmission by aphids:

Adults aphids found transmitting the disease during preliminary investigations were used to study virus vector relationship (non-persistant, semi-persistant or persistant).

2.2.1 (b) Raising of virus free aphids:

Viviparous adults were starved for 2,4,6 and 8h at room temperature in a petridish and then placed upon a detached leaf of an appropriate healthy host in a petridish. The atmosphere inside the petridish was made humid by covering the inner surface of the petridish with wet filter paper. Newly

borne nymphs were transferred to a fresh and healthy test plant. The aphid colonies thus developed were used as healthy colonies of the virus free aphids. The aphids from one plant to other were transferred with the help of moistened tip of camel's hair brush type A, No.1. Colonies of virus free aphids were raised on suitable host plant in cages having wooden frames. The top and two sides of the cage were closed by glass and the remaining sides were closed by wire gauze. A flourescent tube was fixed in the cage to keep the aphids under long day conditions to get the apterous (wingless) aphids. The plants were kept on a zinc-tray and the bottom of the tray was covered with a layer of moist sand to prevent the passing of the aphids through chinks between the tray and the rim of the cage.

2.2. (c). Mode of transmission:

To established the mode of transmission following procedure will be adopted;

Non-persistant:

Pre-acquisition starvation period-1 to 2h.

Acquisition access period – 2 to 5 min.

Inoculation access period – 24h.

Number of aphids / plant – 10

The nymphs were starved for 1 to 2h in a petridish having the inner surface covered with a wet piece of filter paper before an acquisition access period of 2 to 5 min. on the leaf of the diseased plant. After allowing acquisition feeding time, the nymphs in batches of 10 were transferred to each healthy seedling and the plants were covered with Leztz cages for an inoculation access period of 24h., the nymphs after the end of inoculation access were killed by spraying with 0.02 per-cent cypermethrine (insecticide) and the plants were kept in an insect proof glasshouse for the development of symptoms. Back inoculations for each plant were made to an appropriate local lesion host, i.e., *Chenopodium amaranticolor*.

Persistant

Acquisition access period – 24h.

Inoculation access period – 48h.

Number of Aphids / plant – 10.

The virus free aphids, without subjecting them to starvation were allowed 24h acquisition feeding time on diseased leaves. After the completion of acquisition feeding, 10 aphids were transferred to each test plant where they were given an inoculation feeding period. Aphids were killed by spraying an insecticide (cypermethrine 0.02% solution). The test plants were kept in an insect proof glass house to observe

the development of symptoms. Back inoculations from the plants on which aphids were given inoculation feedings were made on a local lesion host, i.e. *C. amaranticolor*.

2.2.2. Transmission by white-flies:

a) Source of virus free white-flies:

White flies (*Bemisia tabaci* Genn) collected from field were caged on a healthy plant of *N. glutinosa* for egg laying. After 10 days the adults were removed from the cage. New born whitefly adults developing after 7-8 days were allowed further multiplication. Insect colonies so raised were virus free and used for transmission studies.

b) Handling of white-flies:

The method described by Rathi and Nene (1974) was used for handling of white flies.

c) Transmission:

Non-viruliferous white flies were allowed acquisition and inoculation access period of 24h each on diseased and healthy plants, respectively. Cypermethrine (0.02%) was sprayed to kill the white flies after inoculation. The test plants were kept for observation of symptoms.

2.2.3. Grafts:

Attempts will be made for side wedge grafting. Infected scions will be grafted on healthy stock and kept under appropriate light and humidity condition to allow successful union which is necessary for transmission.

2.2.4 Dodder:

Seeds of dodder (*Cuscuta reflexa* Roxb. and *C. chinensis* Lam) were germinated on moist filter paper placed in petri-dish and then transferred in 4" clay pots, sterilized with formaline (4%) and containing sterilized soil mixture. When the dodder plants were about 6" long, they were trained on a suitable host plant susceptible to the virus and the host plants (on which the dodder was trained) were inoculated after one week. When the dodder had been established on inoculated plant, a healthy test plant in another pot was placed near the pot (having inoculated plant with dodder established on it) and the tips of the branches of dodder were detached, placed in the axil of the healthy test plant and allowed to establish there. The plants were left as such for about 2 months to develop the symptoms. Back inoculations were made on *C. amaranticolor* to confirm the presence of virus.

2.2.5. *Soil transmission:*

Soil around the naturally infected plants was collected from the field and sieved to remove roots and debris etc. such soil was divided into two parts. One part was filled in a gunny bag and was autoclaved at 15lbs / inch^2 for one hour and the other part of soil was left as such and was filled in pots. Healthy seedlings were sown in pots containing sterilized and unsterilized soil. Plants of both the sets were kept for observation of symptoms in an insect proof glass house. The presence of virus was confirmed by making back inoculations on the test plants.

2.2.6. *Seed transmission:*

To determine seed transmission of the virus, experiments were carried out as follows:

a) Sowing on method:

Seeds were collected from infected and healthy plants and were sown in autoclaved soil in an insect proof glass house. After seedling emergence, the plants were observed till 5-6 weeks and were sprayed with 0.02% cypermethrine, an insecticide at weekly intervals to prevent insect infestation.

b) Infectivity test method:

Leaf tissues of such plants (grown as above) were macerated in 0.01M phosphate buffer (pH-7.0) and the sap obtained was inoculated manually on local lesion host, *C. amaranticolor* to ascertain the presence of virus in them.

3) Host range studies:

Several species of plants, belonging to different families will be screened for the susceptibility to the calendula yellow net virus. Standard inoculum will be used for inoculation of all plants. At a time at least 5 plants of each species will be inoculated and the same number will be kept as a control. Plant at 5-6 leaf stage will be used and all the fully expanded leaves will be inoculated. The inoculated plant will be observed upto two months for the development of symptoms. The time sequence and severity of the symptoms will be noted. Inoculated plants exhibiting no symptom will be kept for about 8 weeks for observation. Back inoculation will be made to a test plants from all the inoculated plants.

4) Virus-vector relationships:

In order to determine the relationship between the virus and the vector, the method would depend on the type of the vector group involved in transmission. However, in general the

variabilities including number of insect per plant, different pre-acquisition starvation periods, varying acquisition and inoculation access periods will be worked out along with effect of moulting of insect on various retention and latent periods in the vector.

5) Effect of different buffers on the infectivity:

Various buffers (Phosphate, borate, citrate, acetate, glycine-NaOH and tris-HCl) at different pH and molarities will be used and tested to find out the most suitable one in which virus infectivity is retained maximum. Young infected leaves will be macerated in a mortar with pestle using a buffer (any of the above mentioned) as extraction medium. The sap obtained after filtrating it through double layered cheese cloth will be inoculated on the leaves of local lesion host following the usual method of inoculation. All buffers will be tested in the same way, and a buffer at a pH and molarity in which virus infectivity is higher will be selected and used regularly as an extraction medium for the virus being used.

6. Virus concentration in different parts of the hosts:

To determine the virus concentration in different parts of the host plant, 10-15 days earlier inoculated plants will be uprooted carefully and washed. The plants will be blotter dried.

Root, stem and leaf tissue will be cut separately into pieces. Equal amount of root, stem and leaf tissue will be macerated separately in mortar and pestle using a suitable buffer. Sap obtained from each sample will be inoculated separately on a local lesion host using usual method of inoculation.

7. Selection of suitable propagation host and an assay host:

To find out a suitable propagation host several plants, susceptible to the virus will be inoculated and showing most prominent symptom will be selected. A plant exhibiting following characters will be selected.

i) Rapid seed germination and fast growth.

ii) Short incubation period of the virus.

iii) Peak concentration of the virus within the short period after inoculation.

iv) Absence of virus inhibitors.

v) More yield of infected tissue with good virus concentration.

Assay of virus will be carried on a local lesion host. To search out a local lesion several commonly used plants will be tested. However, in case of non-availability of a local lesion host, assay tests of the virus will be carried out on a systemic host.

8) Biophysical properties:

To determine the dilution end point, thermal inactivation point and longivity *in vitro*, methods described by Noordam (1973) will be employed.

8.1) Dilution end-point (DEP):

By adding suitable buffer, ten fold dilutions (10^{-1}, 10^{-2}, 10^{-3} .....10^{-8}, 10^{-9}), will be made of the sap obtained from infected leaves of the propagation host after homogenizing them in a mortar with pestle. Each sample will be inoculated on the leaves of the local lesion host following the usual method of sap inoculation. In this way the dilution at which virus loses its infectivity will be determined.

8.2 Longivity in vitro (LIV):

a) **In sap:** The infected leaves of the propagation host will be homogenised in a mortar with pestle, while using a suitable buffer and the homogenate will be filtered through two layers of cheese cloth and the sap thus obtained, will be kept at room temperature (25±5°C). After every 8h interval, a small amount of the sap will be taken and inoculated on the leaves of local lesion host. The process will be counted on the inoculated leaves for each interval and the time after which the virus loses its infectivity will be recorded.

b) **In dried leaves:** The young infected leaves of the propagation host will be cut into small pieces and dried over anhydrous calcium chloride in a dessicator. After 24h interval, such pieces will be homogenised, by using a suitable buffer, in a mortar with pestle. The sap thus obtained after passing through two layered cheese cloth will be inoculated on the leaves of local lesion host. This will be continued upto the time, until the virus loses its infectivity in the tissue dried over anhydrous calcium chloride.

8.3 Thermal inactivation point (TIP):

The sap obtained by the same method mentioned above, will be divided into 12 aliquots of 5ml. each and kept in glass vials. The glass vials will be held in a water bath in such a way that the sap level in the vial is below the water in the bath. The different aliquots will be heated at 40,45, 50.....85, 90°c, for ten minutes and cooled under running tap water, immediately after heating. Each heated aliquot will be inoculated on the leaves of a local lesion host. One aliquot left at room temperature will also be inoculated and will be served as control.

9. Effect of various additives on virus infectivity:

To work out whether the stability and infectivity of the virus will get increased, several additives (Sodium sulfite, DIECA, EDTA, sodium thioglycollate, mercapto ethanol) will be used. Incase, the infectivity get enhanced, the most suitable additive will be selected and routinely added to the medium for virus extraction.

10. Purification:

After selecting a suitable buffer, a propagation host(s), an assay host(s) and biophysical properties, attempts will be made to purify the virus under consideration.

10.1 Clarification of sap:

The infected leaves of the propagation host will be macerated by usual and suitable method and the macerate will be passed through a double layered cheese cloth. The sap thus obtained will be given a low speed centrifugation at 5,000g for 10 minutes. The supernatant (sap) will be subjected to various clarification procedures.

10.1(a) Celite and charcoal: Celite and activated charcoal will be mixed with sap at the rate of 5g per 100ml., either separately or, in combination. When both are to be used 5g of activated charcoal will be mixed with 100ml of sap and after 30

sec. stirring 5g of celite will be added. Shaking will be continued for another half minute. The absorbent will be removed by the following methods.

i) Centrifugation at 2,000 rpm for 5 minutes.

ii) Filteration through Buchner funnel supported by a 2-3mm thick celite pad and filter paper (Whatman No.1) or,

iii) Filteration through a filter paper (Whatman No.1) only in a Buchner funnel.

10.1.(b). Organic solvent: Organic solvents (butanol, ethyalconol, chloroform, carbon tetrachloride and di-ethyl ether) either separately or in combination such as (chloroform – butanol) will be used in two ways for the removal of the extraneous plant material from the infected tissue.

a) By macerating the infected tissue by using a mixture of suitable buffer and organic solvents, or (b) by adding requisite amount of solvent in crude sap obtained after macerating the infected tissue in buffer and filtering through two layers of cheese cloth.

The mixture will be incubated for 30 minutes and then centrifuged at 5,000g for 15 minutes. The aqueous layer will be separated. The effect of solvent on the virus infectivity will be

tested by assaying the aquous layer for active virus content on a local lesion host.

c) **Calcium phosphate gel:** The gel will be prepared by mixing 0.1 M sodium di-basic hydrogen phosphate (Na_2HPO_4. $2H_2O$) and 0.1M calcium chloride (CaCl) in equal volume. The mixture after continuous stirring for 15 minutes will be allowed to settle. Then the supernatant will be decanted. To the remaining precipitate double distilled water will be added and the re-suspended precipitate will again be allowed to settle down. In this way precipitate (gel) will be washed 15-20 times to assure the removal of chloride ions (Cl^-). Ultimately it will be equilibrated with phosphate buffer (0.01M pH-7.0). Such freshly prepared gel will be mixed with sap obtained after low speed (5,000g for 10 minutes) centrifugation of the crude sap, stirred vigorously and centrifuged for 5 minutes at 5000g. The clear supernatant will be assayed for virus activity on local lesion host.

d) **Silver nitrate:** Different volumes of 1 per-cent silver nitrate solution will be added drop by drop to the standard inoculum (1/5) and stirred simultaneously. The mixture will be left at room temperature for 30 minutes and thereafter, centrifuged at 5000g for 15 minutes. The supernatant thus obtained will be bio-assayed on local lesion host for virus infectivity.

Out of clarification methods described above, one will be standardized and used as clarification method in the purification of the virus being studied.

10.2 Concentration of virus:

The sap obtained after clarification treatment as described above will be used for concentration of virus by any of the following methods.

a) ***Differential centrifugation:*** The ultracentrifugation will be worked out in model L7-80. Backman preparative ultracentrifuge using rotor type 50. Normally high speed centrifugation will be done at 50,000g unless otherwise stated. The pellet, thus obtained will be dissolved in a suitable buffer. Low speed centrifugation will be performed at 10,000g in a Remi T-24 centrifuge or any other same type of centrifuge. The number of cycles and the time of centrifugation at different rpm will be carried out keeping in view the stability of the virus and its sedimentation. Activity of different samples in supernatant and the pellet will be assayed on local lesion host.

b) Precipitation:

i) **Poly ethylene glycol (PEG):**

Polyethylene glycol 6,000 will be used for precipitating the virus in clarified sap. Precipitation of the virus will be tried with 2,4,6,8, 10 and 12 per-cent PEG separately. In every case, the variation in salt (NaCl) concentration and its impact on precipitation of the virus will be standardized. After the addition of requisite quantity of PEG and NaCl to the clarified sap, the mixture will be stirred on a magnetic stirrer till both (PEG and NaCl) are dissolved completely and kept in a refrigerator at 4°C to 8°C for 6h to allow complete precipitation.

ii)Ammonium sulphate:

Different quantities (10-40%) of ammonium sulphate $(NH_4)_2$ SO_4 (w/v) will be added to clarified sap (1/1). The mixture will be stirred at 8±2°C in an ice bucket till the $(NH_4)_2SO_4$ cyrstals are dissolved completely. The mixture will then be incubated at 4±1°C for 2 hours and centrifuged at 5,000g for 15 min, to collect the precipitate.

The pellet thus obtained by PEG and $(NH_4)_2SO_4$ precipitation will be dissolved separately in a suitable buffer and re-centrifuged at 5,000g for 5 minutes. Supernatant thus obtained will be assayed on local lesion host.

11. Further purification by density gradient centrifugation:

Concentrated virus samples obtained by the methods described above will be subjected to further purification using density gradient centrifugation (Brakke, 1951, 1960).

Linear sucrose gradient columns will be prepared by layering 7,7,7, and 4ml of 0.01M phosphate buffer (pH 7.0) having 400, 300, 200 and 100 mg sucrose per ml, respectively, in a 1x3" tube. The sucrose solutions of different concentrations will be layered using a pipette with a broad orifice.

The heaviest solution will be layered 1st and the solutions of decreasing concentration will be layered on the top of each other. The column will be used after standing for 24h in a refrigerator usually 2ml. of the virus preparation will be floated on the top of the column and the column will be centrifuged immediately after floating the virus preparation to avoid droplet sedimentation. The column will be centrifuged in SW-25.1 rotor in L7-80 preparative ultracentrigue. The acceleration upto a few hundred rpm will be made gradually. The tubes will be centrifuged for 2½-4hours. After centrifugation the tubes will be examined in a dark room by projecting a beam of light down the tube from the top. The virus zone scattering the light will be removed from the tube by 20

guaze 10 cm long needle bent twice at right angles and attached to a hypodermic syringe.

12. UV-spectrophotometry:

The virus preparation will be examined in Beckmann DU-2 model ultraviolet absorption spectrophotometer to evaluate the different methods of purification and to ascertain the purity of virus isolated.

Ultraviolet radiations are absorbed in a characteristic manner by the virus (nucleo-protein) containing solutions. Absorbance of samples will be studied in UV-range (230-320nm) and graphs will be plotted. Values at A max/min. $A_{280/260}$ and $A_{260/280}$ will be calculated to know the approximate per-centage of nucleic acid.

13. Electron microscopy:

Shape and size of virus particles will be studied in electron microscope.

13.1 Leaf dip method: Method described by Brandes (1964) will be followed for leaf dip preparations. One drop each of 2% potassium phosphotungstic acid (PTA) and uranyl acetate will be placed separately on several formvar coated copper grids having carbon backing. The freshly cut ends of infected leaves will be dipped in the drop for 2-4 seconds. Such grids will be

allowed to dry for sometimes and thereafter examined under electron microscope at various magnifications.

13.2 Procedure with purified virus preparation: A small droplet of purified virus preparation will be placed on formvar coated copper grids having carbon backing, then a small drop of suitable stain (either PTA or uranyl acetate) will be added to the virus suspension. The excess fluid will be absorbed with a small piece of filter paper leaving a very thin film of fluid on the grids, which will be dried at room temperature. Such grids will be examined under electron microscope.

14. Serology:

Specific antigen and antibody reaction is one of the useful techniques either for assigning the virus to a particular group or to differentiate it at the strain level. Antisera to the virus under consideration will be prepared for the identification of the virus as well as for testing the latent infection in certain hosts. Besides, it would also be useful for ascertaining the seed transmissible nature of the virus through routine serological methods or by enzyme linked immuno-sorbent assay (ELISA).

14.1 Raising of antisera: Young healthy rabbits, approximately 3lbs. in weight will be used for production of antisera. The

purified or partially purified virus preparation will be used as antigen.

To work-out the effect of injection on the formation of antibodies as well as titre of the antiserum, antigen (virus preparation) will be injected intravenously or intramuscularly or in both ways.

The antigens will be administered intravenously through the marginal ear vein of the rabbit using a clinical syringe with a fine needle. Five to seven weekly injections of virus preparation of 2ml. each will be administered intravenously through the marginal vein of the ear. For intramuscular injections, antigens will be emulsified with an equal volume of Freund's incomplete adjuvant. The injections of the virus adjuvant mixture of 3ml. each at an interval of 2 weeks will be administered intramuscularly in thigh of the same rabbit which has been given intravenous injections at antigens.

Test bleedings will be made several times from the ear of the rabbit at different intervals after the administration of last intramuscular injection to check the antibody titre in serum. After the titre has reached its maximum, the immunized rabbits will be finally bled by giving a sharp incision on the marginal vein of the ear, which has not been used for injecting the antigen. About 10-15 ml. of the blood will be collected and

allowed to clot at room temperature (25±5°C) for 2 hours and kept overnight in a refrigerator. Serum containing antibodies (antiserum) will there after be separated and centrifuged at 1,000 rpm for 5 minutes to remove fibron, blood cells etc. The straw yellow coloured antiserum will be collected and stored for serological studies.

To identify the virus under investigation upto group or strain level, the Ouchterlony's double diffusion test (Ouchterlony, 1962) will be performed.

14.2 Ouchterlony double-diffusion test: One per-cent agar will be prepared in 0.85% saline containing 0.1-0.2% sodium azide. Suitable amount of agar will be poured into sterilized petri dishes so as to get a 2-3mm thick agar bed. Using a cork borer, 5mm. diameter well will be made into the agar and the cut portions of agar will be removed by aspiration. The distance between two wells will be kept 5mm. apart. The central well will be filled with antiserum. The remaining wells will be loaded with various dilutions of antigens made in physiological saline (0.85% NaCl solution) and the clarified sap from healthy plant. Such treated petri-dishes will be incubated at room temperature and observed for formation of precipitation lines.

14.3 Precipitation in tubes: Equal proportions of antisera and antigen after making 2 fold dilutions in 0.85% saline will be

mixed together in serological tubes (6x1cm) and incubated at 37°C in a water bath. The formation of precipitate and its intensity will be observed using a magnifying lens.

15. Isolation of nucleic acid:

Phenol detergent method will be used to isolate the nucleic acid of viruses. To a 2.5ml of purified virus preparation will be added 0.05ml of 6% sodium dodecyl sulphate and 2.5ml of water saturated phenol. The phenol used will be redistilled and stored at 10°C after adding distilled water. The mixture will be stirred in a glass tube on a magnetic stirrer for 10 minutes and then centrifuged for 5 minutes at 3,000 rpm in a clinical centrifuge. The mixture will separate into two layers, the upper aqueous layer and the lower phenol layer containing sodium dodecyl sulphate. The top aqueous layer will be drawn with a pipette. To the lower phase 2.5ml of 0.01M phosphate buffer (pH 7.0) will be added and stirred for 10 minutes and then centrifuged for 5 minutes at 3,000rpm. The aqueous phase will be drawn off and pooled together with the aqueous phase obtained at previous step and stirred for 10 minutes with an equal volume of phenol followed by centrifugation. The aqueous phase will be extracted once more with half the volume of phenol. Traces of phenol will be removed from the aqueous phase by extraction with ether. The nucleic acid will

be precipitated by the addition of 2ml. of ice-cold ethanol to the solution. The precipitate will be pelleted out by centrifugation for 15 minutes at 7,500 rpm. The pellet will be suspended in 0.01M phosphate buffer (pH 7.0) and centrifuged for 15 minutes at 10,000 rpm to remove any insoluble material present in the precipitation, and the supernatant thus obtained will be tested for infectivity and type (RNA or DNA) of the nucleic acid.

15.1 Infectivity of viral nucleic acid: The infectivity of viral nucleic acid will be assessed by inoculating the nucleic acid preparation on local lesion host. Several dilutions of nucleic acid preparation will be made and inoculated on the local lesion host and the number of local lesions developed will be compared with the corresponding dilutions of the virus preparation.

15.2 Type of nucleic acid: It is well known that RNA and DNA differ in their chemical composition with respect to the base and sugar involved in their composition. RNAs are known to contain ribose sugar and uracil base (other three bases being adenine, cytosine and guanine) while DNAs contain thymine (other three bases are same as in case of RNA) and deoxyribose sugar.

Thus, test will be performed to study the type of sugar. Diphenylamine test for deoxyribose or orcinal test for ribose

sugar will be used for ascertaining the type of nucleic acid in virus under investigation.

16. Studies on proteins of the virions:

Attempts will be made, while using standard methods to determine the approximate molecular weight of proteins associated with the virions.

Chapter 4
Results

1. Natural Symptoms:

Naturally infected plants of Calendula *(Calendula officinalis* L.) showed severe yellow net mosaic symptom on leaves. At advanced stage of infection, the infected plants showed reduction in leaf size with retarted vegetative growth of plant. Severely affected plants produced few flowers of small size (fig. 1a).

2. Transmission:

a) ***By Sap***: The virus causing severe yellow net symptoms in Calendula was readily transmitted by sap extracted in 0.01M phosphate buffer (pH-7.0) from Calendula to Calendula, *Nicotiana* sp. and other susceptible hosts. The transmission of the disease, using carborundum (500 mesh) as an abrasive was 90-100% by sap. In subsequent studies, the infected Nicotiana leaves were macerated in 0.01M phosphate buffer pH-7.0 (1:2 w/v), and the sap thus obtained used as standard inoculum (SI).

3. Host range and symptomatology:

To determine the host range of calendula yellow net virus various species and cultivars of plants belonging to different families were inoculated mechanically with standard inoculum.

To ascertain the presence or absence of the virus, back inoculations from all inoculated plants were made on *C.amaranticolor.* and the plants inoculated developed symptoms as described below and the virus was recovered on back inoculation in most of the cases.

Asteraceae:

***Calendula officinalis* L.**

Systemic symptoms appeared after 14-15 days of inoculation on the new emerging leaves in the form of vein yellowing followed by yellow net. In severe condition the leaves became deformed and reduced in size.

Apiaceae:

***Daucus carota* L. cv. Pusa Kesar:**

Systemic symptoms appeared after 12-14 days of inoculation on the new coming leaves in the form of mosaic followed by yellow net. In severe condition the leaves shows distortion and retarded growth of plant (fig. 2a).

Fig.1a. Natural symptoms on C. officinalis leaf showing yellow net symptom (Right) healthy (left).

Fig.2a. Daucus carota L.cv. Pusa Kesar showing systemic symptoms in the form of mosaic followed by yellow net.

Fig. 3a. C. amaranticolor Coste & Reyn, showing local lesions in the form of pin head. (Right two leaves) & healthy (left leaf).

Fig. 3b. C. amaranticolor Coste & Reyn plant showing pin head type of local lesions.

Chenopodiaceae:

***Chenopodium amaranticolor* Coste and Reyn.**

Pin head type of local lesions were formed after 5-6 days of inoculation and a typical yellow net type of systemic symptoms were developed after 20-25 days of inoculation (fig. 3a,b & c).

Solanaceae:

***Nicotiana tabacum* cv. Harrison Special:**

Necrotic leaves were formed after 10-12 days on the older leaves and new emerging leaves show yellow net type of symptoms after 20-25 days and plant shows stunted growth and reduced leaf size (fig. 4a & b).

Nicotiana tabacum:

Severe mosaic type of systemic symptoms were developed on new emerging leaves after 14-16 days of inoculation. The whole plant remained stunted and the dark and light areas appeared with deformed leaves (fig. 5a).

***Nicotiana tabacum* cv. Anand-2 Bidi Type:**

Systemic infection were observed after 12-13 days of inoculation in the form of mosaic (fig. 6a).

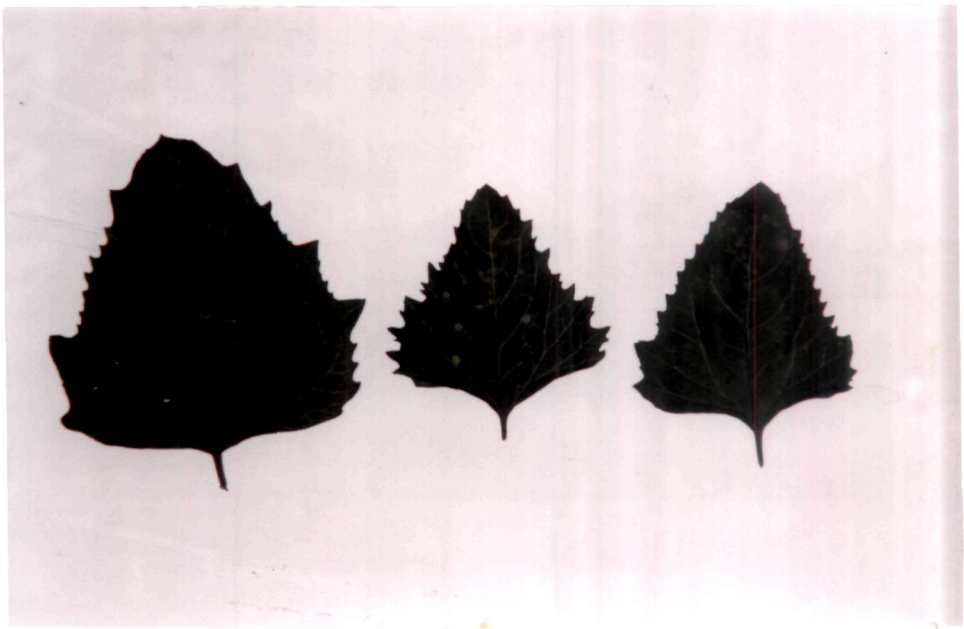

Fig. 3c. C. amaranticolor Coste & Reyn, systemic symptoms in the form of yellow net. (Right two leaves) & healthy (left leaf).

Fig 4a. Nicotiana tabacum cv. Harrison's Special showing necrotic local lesions.

Fig. 4b. Nicotiana tabacum cv. Harrison's Special leaf showing yellow net type of systemic symptoms.

Fig. 5a. Nicotiana tabacum, systemic symptoms in the form of severe mosaic followed by dark and light areas on leaf.

Fig. 6a. Nicotiana tabacum cv. Anand-2 Bidi Type. Systemic symptoms in the form of mosaic (right leaf) & healthy (leaf leaf).

Non-hosts:

No symptoms (local / systemic) were produced and no virus could be recovered on back inoculation to *C. amaranticolor* from the following plant species and cultivars. These plants were kept under observation for 6 weeks after inoculation.

Amaranthaceae:

Amaranthus caudatus L.

Apiaceae

Coriandrum sativum L.

Petroselenium crispum Hill cv. Imperial Curie.

Astraceae:

Tagetes erecta L.

Ageratum maxicanum Sims.cv. Blue Mink

Dahlia pinnata Cav. cv. Dwarf Mixed.

Helianthus annus L.

Zinnia elegans Jacq. cv. Suttons Giant Double Mixed.

Brassicaceae:

Brassica compestris L.

B. oleracea L. var. botrytis cv. Snow Ball.

cv. Pusi

B. rapa L..cv. Purple Top White.

Raphanus sativus L.cv. Bombay Red.

cv. Chinese Pink.

cv. Pusa Himani.

Chenopodiaceae:

Chenopodium album L.

C. murale L.

C. quinoa L.

Beta vulgaris L.

Spinacia oleracea L.

Cucurbitaceae:

Citrullus vulgaris Schrad var. fistulosus cv. Dilpasand.

Cucumis melo var. utilissimus cv. Lucknow Sweet.

Cucumis sativus L. cv. Pointsetee SLG

cv. Poona Kheera

Momordica charantia L. cv. Poona Long Green.

cv. Coimbatore Long.

Cucurbita maxima Duch. cv. Large Red.

Luffa cylindrical (L.) Roem.

Liliaceae:

Alium cepa L.cv. Red Globe.

Malvaceae:

Abelsmoschus esculentus (L.) Moench cv. Pusa Sawani

Papilionaceae:

Pisum sativum L.

Lathyrus odoratus L.cv. Mixed.

Solanaceae:

Capsicum annuum L.cv. Cluster Suryamukhi

Nicotiana tabacum L.cv. Bhopali Pakra.

cv. CTRI special type FCV.

cv. Jayasri type FCV.

cv. Samsun type Turkish

N. clevelandii Gray.

N. debneyi Domin.

N. glutinosa L.

N. longiflora Cav.

N. megalosiphon Heurek and Mucller

N. occidentalis Wheeler

N. plumbaginifolia Viv.

N. rustica Schrank.

Datura metel L.

Lycopersicon esculantum L.

Solanum melongena L.cv. Black Beauty

cv. Pusa Purple Long

S. nigrum L.

Petunia hybrida Vilm.

Verbenaceae:

Verbena hybrida L.cv. Tall Mixed.

4. Selection of local lesion host:

Two local lesion hosts of the calendula yellow net virus (CYNV) viz. *Chenopodium amaranticolor*, Coste and Reyn and *Nicotiana tabacum* L.var. Harrison special were compared to select the most suitable one. The inoculum prepared from infected *N. tabacum* was inoculated to the above hosts and local lesion were counted 3-4 days after inoculation.

Table 4.1. Comparative study of different local lesion hosts of calendula yellow net virus (CYNV).

Local lesion hosts	No. of local lesions / leaf*
Chenopodium amaranticolor	25
Nicotiana tabacum L.var. Harrison special	28

* Average number of local lesions / leaf based on three experiments with three plants having 6 leaves each.

The lesion produced on *C. amaranticolor* were necrotic and were easily countable and consistent. On the basis of these characteristic *C. amaranticolor* was chosen as the assay host and test plant for the present virus. (Table 4.1)

5. Properties of the virus in plant sap:

Parameters such as thermal inactivation point (TIP), dilution end point (DIP), and Longivity *in vitro* (LIV) in the identification of plant viruses provide information about the best environment in which to keep the virus and maintain its infectivity. Although, these studies have restricted value (Ross, 1964) but are of great help in determining the procedure for purification of the virus and in its characterization.

To study these properties, experiments were carried out using *Nicotiana tabacum* as donor host and *C. amaranticolor* as assay host of the virus. Three experiments of each property were performed using *C. amaranticolor* as an assay host.

a) Thermal inactivation point (TIP):

The virus in crude sap was found to be infectious after being heated for 10 min. at 60°C. but was found to be inactive after being heated at 65°C for 10 min. (Table 5.1) Thus, the thermal inactivation point (TIP) of the virus lies between 60°C and 65°C.

b) Dilution end point (DEP):

The virus in crude sap was found to be infectious at a dilution of 10^{-4} but no local lesion were observed when the sap was diluted to 10^{-5} (Table 5.2). Therefore, the dilution end point of the virus is between 10^{-4} and 10^{-5}.

C) Longevity *in vitro* (LIV):

The crude sap from a propagation host was obtained and divided into two parts. One was kept at room temperature (25±5°C) and the other in a refrigerator (4°C). Each sample was assayed separately on *C. amaranticolor* after a specific period of storage (Table 5.3). The virus was found to be remain infective at room temperature for 56h and at 4°C for 96h.

Table 5.1. Effect of temperature on the stability of calendula yellow net virus (CYNV).

Temperature	No. of local lesions / leaf*
Room temperature (25±5°C)	28
40	22
45	18
50	14
55	08
60	04
65	00
70	00
75	00
80	00
85	00
90	00

Table 5.2: Effect of dilution on the infectivity of calendula yellow net virus (CYNV).

Dilution	No. of local lesions / leaf*
Undiluted	28
10^{-1}	24
10^{-2}	14
10^{-3}	08
10^{-4}	04
10^{-5}	00
10^{-6}	00
10^{-7}	00
10^{-8}	00

*Average no. of local lesions / leaf based on three experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

Table 5.3: Effect of storage on the infectivity of the virus causing yellow net disease on Calendula in plant sap at room temperature and at 4°C..

Storage in hours	Average no. of local lesions / leaf*	
	At room temperature	At 4°C
00	28	28
08	20	24
16	16	20
24	14	16
32	10	12
40	08	08
48	04	06
56	02	04
64	00	02
72	00	02
80	00	02
88	00	01
96	00	01
104	00	00
112	00	00
120	00	00
128	00	00
136	00	00
144	00	00
152	00	00
160	00	00

*Average no. of local lesions / leaf based on 3 experiments with 3 plants of *C. amaranticolor* having 6 leaves each.

6) Selection of propagation host:

Six hosts of the virus causing yellow net symptoms in calendula viz. *Calendula officinalis* L., *Daucus carota* L.cv. Pusa Kesar, *Chenopodium amaranticolor* Coste and Reyn. *Nicotiana tabacum* cv. Harrison special, *Nicotiana tabacum*. *Nicotiana tabacum* cv. Anand-2 Bidi Type were compared with regard to virus concentration at different times after inoculation. At different intervals (after inoculation), the plants were assayed for virus concentration during *C. amaranticolor* as a local lesion host. The results showed (Table 6.1) that the virus reached its maxtimum concentration in *C. officinalis* L. and *D. carota* L. Pusa Kesar after 16 days of inoculation. In *C. amaranticolor* L. and *N. tabacum* cv. Harrison Special, virus reached its maximum concentration after 14 and 12 days respectively. Virus reached its maximum concentration, 12 days after inoculation in *N. tabacum* and in *N. tabacum* cv. Anand-2 Bidi Type.

Thus *N. tabacum* and *N. tabacum* L.cv. Harrison Special were selected and routinely used as propagation host for purification due to sufficient availability of plants, early growth and maximum concentration of the virus was attained in shorter period.

Table 6.1: Concentration of calendula yellow net virus in different hosts at different intervals after inoculation.

Propagation Hosts	Average no. of local lesions / leaf*									
	days after inoculation									
	2	4	6	8	10	12	14	16	18	20
Calendula officinalis L.	00	00	00	02	08	14	18	22	20	19
Daucus carota L.cv. Pusa Kesar	00	00	00	03	07	12	16	20	19	17
Chenopodium amaranticolor . Coste and Reyn.	00	00	00	03	10	16	22	20	19	18
Nicotiana tabacum cv. Harrison Special.	00	00	02	10	22	26	24	22	21	20
Nicotiana tabacum	00	02	08	14	22	30	28	26	25	24
N. tabacum L.cv. Anand-2 Bidi Type	00	03	06	12	18	26	25	23	22	20

*Average number of local lesions / leaf based on three experiments with three plants having 6 leaves each.

7) Effect of buffers:

Effect of acetate, boric acid borax, citrate, citrate phosphate and potassium phosphate buffers at different pH values were compared for extraction of yellow net virus of calendula from infected leaves of *N. tabacum* plants.

Results presented in the table 7.1 indicated that the maximum infectivity was obtained with phosphate buffer (pH 7.0).

8) Effect of molarity:

Attempts were made to determine the most suitable molarity (ionic strength) of potassium phosphate buffer (pH 7.0) for virus infectivity. At different molarities the virus infected leaves of *N. tabacum* were macerated in phosphate buffer (pH7.0) separately. The samples were assayed on local lesion host, *C. amaranticolor* to compare virus infectivity.

It is clear from the table 8.1 that the extraction of infected leaves in 0.01M potassium phosphate buffer at pH 7.0 gave the highest infectivity.

Thus for the extraction of virus from infected tissue and maintaining the extracts at different stages of purification, 0.01M potassium phosphate buffer (pH 7.0) was found to be suitable at which virus infectivity was highest.

Table 7.1 Effect of various buffers at different pH levels on the infectivity of virus

Buffers	pH	No. of local lesions / leaf*
Acetate	6.0	18
	5.5	20
	5.0	22
	4.5	16
	4.0	08
Boric acid borax	9.0	19
	8.5	20
	8.0	22
	7.5	23
	7.0	18
Citrate	7.0	22
	6.5	20
	6.0	18
	5.5	12
	5.0	08
	4.5	05
Citrate phosphate	7.5	18
	7.0	14
	6.5	16
	6.0	18
	5.5	12
Potassium phosphate	8.0	14
	7.5	18
	7.0	28
	6.5	24
	6.0	20

Table 8.1. Effect of molarity of potassium phosphate buffer pH 7.0 on the infectivity of virus.

Molarity	No. of local lesions / leaf*	Relative infectivity
0.001M	22	73.33
0.05M	27	90.00
0.01M	30	100.00
0.1M	26	86.66
0.5 M	27	90.00
1.0M	18	60.00

*Average number of local lesions / leaf based on three experiments with three plants of *C. amaranticolor* having 6 leaves each.

9) Effect of additives on the infectivity of virus:

Table 9.1 shows that the combination of Thioglycollic acid (0.1%) and Ethylenediamine tetra acetic acid (EDTA 0.1M) was better to maintain the infectivity of the virus at the time of extraction. These additives were included in phosphate buffer. Phsophate buffer (0.01M, pH-7.0) containing 0.1% Thioglycollic acid and 0.1M EDTA was used to macerate infected tissue (1:2w/v).

10) Effect of organic solvents on the infectivity of calendula yellow net virus:

Sap extracted from infected *N. tabacum* in extraction buffer (0.01M, pH-7.0) containing 0.1% Thioglycollic acid and 0.1M EDTA was mixed with different organic solvents (Butanol, Ethyl alcohol, Chloroform and Carbon tetrachloride) either alone or in different combination and incubated for 15 min. at 4°C. Samples were centrifuged for 10 min. at 5,000 rpm. The aqueous layer was separated and assayed on *C. amaranticolor*. Sap extracted from infected *Nicotiana tabacum* leaves in extraction buffer only was used as control.

Result presented in Table 10.1 reveal that Ethyl alcohol, chlorofom, carbon tetra-chloride and their mixture with each other (1:1) showed adverse effect on virus infectivity when added after extraction of sap but butanol added to the extract showed increased in infectivity.

Table 9.1: Effect of additives on the infectivity of calendula yellow net virus (CYNV)

Additives	No. of local Lesions / leaf*	Relative Infectivity (%)
Control	30	100
Thioglycollic acid (0.1%)	26	86.66
Ethylenediamine tetra acetic Acid (EDTA 0.1%)	22	73.33
Sodium sulphite (0.1%)	19	63.33
Thioglycollic acid (0.1%) + EDTA (0.1M)	33	110.00
EDTA (0.1M) + Sodium sulphite (0.1%)	25	83.33
Thioglycollic acid (0.1%)+ Sodium sulphite (0.1%)	29	70.00

Table 10.1: Effect of some organic solvents on the infectivity of calendula yellow net virus (CYNV).

Organic solvents	Final %age of Organic solvent	Average No. of local lesions / leaf*
Control	-	30
Chloroform	20	24
Ethyl alcohol	20	20
Butanol	20	34
Carbon tetrachloride	20	28
Chloroform + Butanol (1:1)	20	26
Butanol + Ethyl alcohol (1:1)	20	27
$CC1_4$ + $CHCl_3$ (1:1)	20	27
Butanol + $CHCl_3$ (1:1)	20	24
Ethyl alcohol + $CHCl_3$ (1:1)	20	18

*Average number of local lesions / leaf based on three experiments with three plants of *C. amaranticolor* having 6 leaves each.

Chapter 5
Discussion

The virus causing calendula yellow net disease on calendula was observed in the month of February (2000) in the garden of Harduaganj, Aligarh. The symptoms observed were a beautiful network of yellowing on the leaves. The infected plants were shorter and the growth was retarded. The flowers as such were not as healthy and compact as on uninfected plants and the colour and brightness of the flowers were also greatly affected. The culture of the virus causing yellow net of calendula was maintained in an insect proof glass-house by a single lesion inoculation on to many species of *Nicotiana* as well as calendula itself.

From the pure culture of CYNV the preliminary studies on this virus were carried out e.g. biophysical properties, host range studies and effect of various buffers with different molarity etc. An attempt has also been done to transmit the virus by many aphid species although mechanical inoculation were successful in most of the time.

Whatever studies presented in this dissertation never revealed the identity of the virus causing yellow net disease of calendula. However, the disease has much resemblance with the virus causing mosaic disease on *Calendula officinalis* (Padma *et al.*, 1975) as the plants used in host range studies were common e.g. *Nicotiana glutinosa* and *C. amaranticolor* in

both the cases. In the case of calendula yellow net disease *Myzus percsicae* was able to transmit the virus while the transmission by *Aphis gossypii* is yet to be performed.

Nothing can be concluded by performing the above studies unless electron microscopy and serological studies will be done so the present isolate may not be placed in any particular group except that it may be a strain of already described virus on a new virus itself.

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